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A direct effect of activated human p53 on nuclear DNA replication

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p53 is a transcriptional activator and repressor, but recent evidence suggests that some of its many biological functions may not be dependent on transcription. To determine whether p53 exerts a direct influence on nuclear DNA replication, purified human p53 was added to a transcription-free DNA replication extract from *Xenopus* eggs. Full-length human p53 that inhibits SV40 DNA replication *in vitro* had no effect on nuclear DNA synthesis in the *Xenopus* system. In contrast, a C-terminal truncated form of p53 (p53Δ30), which is constitutively active for DNA binding and similar to an alternately spliced form found *in vivo*, showed a concentration-dependent inhibition of DNA replication in both the soluble SV40 system and eukaryotic nuclei. This inhibition occurred primarily at initiation of DNA synthesis. Oxidation of p53Δ30, which eliminates DNA binding activity, also abrogated the protein's ability to inhibit nuclear DNA synthesis. The p53 binding DNA consensus sequence enhanced rather than competed away inhibitory activity of p53Δ30. Therefore, p53 that is constitutively active for DNA binding can inhibit nuclear DNA replication in the absence of transcription. This inhibition may require binding of p53 to DNA, in addition to interactions between p53 and proteins of the replication complex.

Key words: DNA replication/p53/*Xenopus* cell-free extracts

Introduction

p53 is a tumour suppressor protein that is important in maintaining genomic integrity. The protein has been postulated to act as a 'guardian of the genome' (Lane, 1992), monitoring the state of the cell's DNA. p53 normally has a short half-life and is present in undetectably low amounts in normally dividing cells, but it is induced to high levels on DNA damage, resulting in arrest of cell growth and division (Kasten *et al.*, 1991, 1992; Lu and Lane, 1993). The importance of p53 in this context can be inferred from the observation that loss or mutation of p53 is found in more than half of all human tumours (Mulligan *et al.*, 1990; Nigro *et al.*, 1989). The high levels of p53 induced in response to DNA damage correlate with arrest at the G₁ stage of the cell cycle (Kasten *et al.*, 1992) and this has generally been attributed to the transcriptional activity of p53 (Fields and Yang, 1990). The hypothesis of an indirect transcriptional effect of p53 on the cell

cycle has been strengthened by recent reports that p53 transcriptionally induces a protein p21/WAF-1 that inhibits the activity of the cell cycle regulatory cyclin-dependent kinases in G₁ (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Serrano *et al.*, 1993; Xiong *et al.*, 1993). Additionally, p21 has been found to bind to PCNA, the DNA polymerase δ auxiliary factor, and p21 itself can prevent synthesis of SV40 DNA *in vitro*, presumably via this interaction (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994; Warbrick *et al.*, 1995).

However, various lines of evidence suggest that p53 can also act to suppress growth by non-transcriptional mechanisms. Some mutant p53 proteins that have lost transcriptional transactivation capacity retain their ability to arrest cell growth to varying extents (Zhang *et al.*, 1993a,b; C.A.Midgley, unpublished results). The domains of p53 involved in transcriptional activation (N-terminal) may differ from those required for transcriptional repression (C-terminal) (Sang *et al.*, 1994; Subler *et al.*, 1994), suggesting that these two phenomena are separable and that growth arrest or tumour suppression may not require the transactivation capacity of p53 (Crook *et al.*, 1994). Importantly, it has recently been demonstrated that p53-dependent apoptosis of somatotrophic progenitor cells in response to X-rays occurs in the absence of new RNA or protein synthesis (Caelles *et al.*, 1994). Conversely, loss of growth suppressor function has been reported in some p53 mutants which retain transcriptional activity (Zhang *et al.*, 1994). However, discrepancies do exist in the literature and other authors find a very strong correlation between transcriptionally active p53 and the ability of the protein to suppress cell growth (e.g. Reed *et al.*, 1993; Pietenpol, 1994).

It has long been known that p53 can directly prevent viral DNA synthesis by binding to and inactivating SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979; Braithwaite *et al.*, 1987; Wang *et al.*, 1989; Friedman *et al.*, 1990) and p53 may compete with DNA polymerase α for binding to T antigen (Gannon and Lane, 1987). In DNA synthesis, T antigen functions as a helicase to promote strand unwinding at the replication fork, and p53 down-regulates this activity (Sturzbecher *et al.*, 1988). Therefore, it is conceivable that p53 interacts with cellular proteins analogous to T antigen to prevent DNA replication under unfavourable conditions, e.g. when damage to the genome has been sustained. Following from this prediction, various cellular proteins have been isolated that compete with T antigen for p53 binding (Takimoto *et al.*, 1994) and that bind to a conformation-sensitive domain of p53 (Maxwell and Roth, 1993; Iwabuchi *et al.*, 1994). More specifically, Dutta *et al.* (1993) have shown that p53 interacts with the single-strand DNA binding protein RP-A and that this association is sufficient to prevent replication of viral DNA in a soluble *in vitro* system.

However, it has been noted that mutants of p53 that no longer arrest cell growth are still able to bind to RP-A, and p53 might even enhance DNA replication, since the transactivation domain of p53 fused to the DNA binding region of Gal4 has been found to stimulate polyomavirus or bovine papillomavirus DNA replication (He *et al.*, 1993; Li and Botchan, 1993). On the basis of these observations, a model has been proposed where p53 binds to cellular origins of replication and there may interact with key replication proteins to block entry into S phase or to direct S phase cells into apoptosis (Pietenpol and Vogelstein, 1993). In addition, by virtue of its non-specific nucleic acid binding properties, p53 has been found to promote re-annealing of DNA and RNA strands (Oberosler *et al.*, 1993; Brain and Jenkins, 1994), thus acting as an anti-helicase, a property that would suggest the protein should be active in preventing nuclear DNA synthesis. However, an effect on nuclear, as opposed to viral, DNA replication has not previously been experimentally addressed, possibly because of the confusion between the indirect, transcriptional contribution and a direct role.

In this paper we have therefore investigated whether p53 has a direct role in arresting nuclear DNA replication. In order to distinguish between a transcriptional role and a more direct effect of p53 in causing growth arrest, we examined the effect of purified p53 on the replication of DNA in cell-free extracts of *Xenopus* eggs. Such extracts support the initiation and elongation stages of DNA replication in a manner that is regulated temporally (Blow and Watson, 1987; Hutchison *et al.*, 1987) and spatially (Hutchison and Kill, 1989; Mills, *et al.*, 1989; Cox and Laskey, 1991), but they do not support transcription (Bachvarova and Davidson, 1966).

Activity of mammalian p53 *in vivo* is regulated by a variety of mechanisms, including protein degradation, phosphorylation, redox, oligomerization and allosteric modification of the C-terminus (Hupp *et al.*, 1992, 1993; Hupp and Lane, 1994). An alternately spliced form of murine p53 (p53_{as}) has been described in normal (Han and Kulesz-Martin, 1992) and transformed cells (Arai *et al.*, 1986; Milner *et al.*, 1993). This protein, which represents 25–33% of total cellular p53, is preferentially expressed in G₂ of the cell cycle under normal conditions, but becomes preferentially expressed in G₁ on treatment with actinomycin D (Kulesz-Martin *et al.*, 1994), both being occasions when DNA synthesis must be suppressed. p53_{as} is nine amino acids shorter at the C-terminus than the major p53 and therefore would not be subject to the same allosteric modifications as the major p53. In this study, therefore, two different forms of p53 were compared for activity; full-length wild type p53 (wtp53) and a 30 amino acid C-terminal truncation (p53Δ30). wtp53 has latent sequence-specific DNA binding capacity that can be activated, for example by phosphorylation by casein kinase II at the C-terminus (Hupp *et al.*, 1993; Meek *et al.*, 1990), whereas p53Δ30 is constitutively active for DNA binding (Hupp *et al.*, 1992). Correlation between the new structural models of p53 (Cho *et al.*, 1994; Clore *et al.*, 1994) and accumulated mutational data (Hollstein *et al.*, 1992) suggests that DNA binding is critical for the tumour suppressor function (Friend, 1994). It is conceivable that effective mechanisms to inactivate the DNA binding or growth suppressing properties of p53 exist in

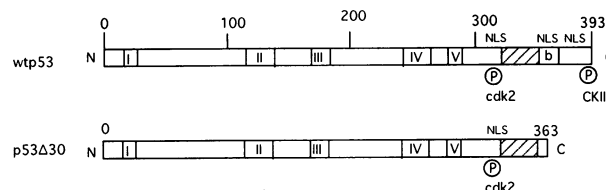


Fig. 1. wtp53 and p53Δ30 structure. Full-length human p53 consists of 393 amino acids. Within the C-terminus are found nuclear localization sequences (NLS), an oligomerization domain (hatched box, amino acids 320–360), a basic region (b) and sites which can be phosphorylated by the cell cycle kinase cdk2 and casein kinase II (CKII). In p53Δ30, the C-terminal 30 amino acids have been deleted, such that one NLS and the oligomerization domain are conserved, but the CKII phosphorylation site is lost. This protein is constitutively active for sequence-specific DNA binding (Hupp *et al.*, 1992).

the activated *Xenopus* egg extract in order to permit the very rapid synchronous cell cycles of alternating S and M phases of early amphibian embryos. Therefore, it was important to employ these two forms of the protein, one (wtp53) that retains the C-terminus regulatory domain and is susceptible to putative regulatory factors in the *Xenopus* egg extract and the other, p53Δ30, that should be relatively resistant to allosteric modulation. Here we show that human p53Δ30 inhibits nuclear DNA replication in a reversible, concentration-dependent manner, while full-length p53 with inducible DNA binding activity has no effect on nuclear DNA replication. The inhibitory activity of p53Δ30 is found to be ablated by oxidation of the protein. Importantly, this is the first demonstration that activated p53 can inhibit true eukaryotic nuclear, rather than viral, DNA replication in the absence of transcription.

Results

p53Δ30 blocks nuclear DNA replication

To determine whether p53 affected nuclear DNA replication, purified recombinant human protein, either full-length wild-type (wt) p53 or p53Δ30 lacking the C-terminal 30 amino acids (Figure 1), was added to *Xenopus* egg extract containing sperm chromatin, and DNA synthesis was analysed by incorporation of [α -³²P]dATP. Figure 2A shows that replication of sperm nuclei was significantly inhibited by 20 ng/μl p53Δ30 and completely abolished at 40 ng/μl, whereas full-length wtp53 that had been purified under identical conditions did not appreciably affect the levels of replication of sperm nuclei. A mutant form of full-length p53 (His273) that lacks growth suppressing activity *in vivo* and DNA binding capacity *in vitro* (Halazonetis *et al.*, 1993) does not affect replication of sperm nuclei in this system (data not shown). Immunoblots of these samples probed with the N-terminal anti-human p53 antibody DO1 (Figure 2B) show that wtp53 and p53Δ30 were both equally stable during the course of the experiment. In contrast, wtp53 and p53Δ30 both inhibited replication of SV40 DNA in a HeLa cell extract (Figure 2C), demonstrating that the wtp53 is functionally active (Wang *et al.*, 1989; Friedman *et al.*, 1990). α -amanitin added to the replication extract neither decreased nuclear DNA synthesis in the presence of wtp53 nor did it relieve the inhibition imposed by p53Δ30 (data not shown), verifying that p53Δ30 blocks nuclear DNA replication in the absence of transcription.

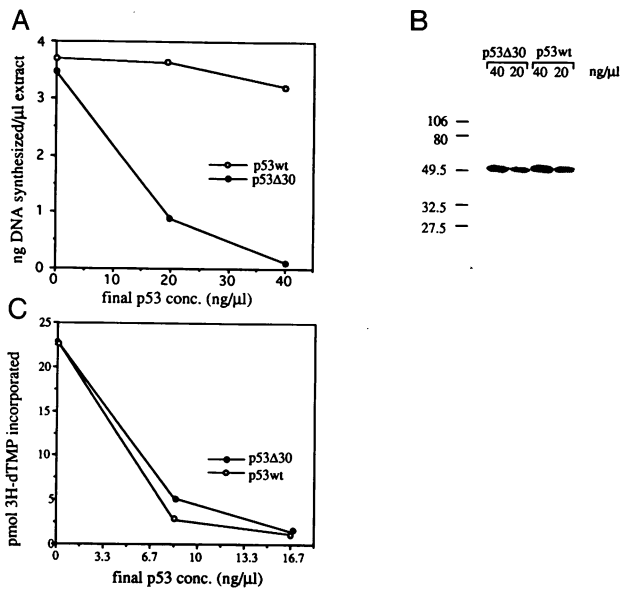


Fig. 2. p53Δ30 but not full-length wtp53 inhibits nuclear DNA replication. (A) Inhibition of nuclear DNA replication by p53Δ30 (closed circles) is concentration-dependent, whereas wtp53 (open circles) does not prevent replication. (B) Immunoblot probed with anti-human-p53 monoclonal antibody DO1 following 3 h incubation of wtp53 or p53Δ30 in *Xenopus* egg extract [samples taken from the experiment shown in (A)], demonstrating that full-length and truncated p53 are equally stable in this system. (C) Both wtp53 (open circles) and p53Δ30 (closed circles) inhibit SV40 T antigen-dependent replication of circular plasmid DNA containing the SV40 origin of replication, as previously reported (Friedman *et al.*, 1990; Wang *et al.*, 1989).

p53Δ30 inhibits initiation of DNA replication

Is the replication block by p53Δ30 exerted at the level of initiation or elongation during DNA synthesis? p53Δ30 was added to egg extract at different times during replication and the amount of DNA synthesis measured at the time of p53 addition (filled columns) or after a total of 3 h incubation (hatched columns). From Figure 3A it is apparent that the inhibitory effects of p53Δ30 are greatest if the protein is added within the first 15 min of incubation. It is during this period that sperm chromatin decondenses and is assembled into intact nuclei, and that initiation of DNA replication takes place (Blow and Laskey, 1986; Blow and Watson, 1987). Immunofluorescence microscopy analysis (data not shown) revealed that nuclei were assembled normally in the presence of either wtp53 or p53Δ30 and that the proteins became localized within nuclei. Therefore, the observed inhibition was not due to a failure in nuclear assembly.

A gradual decrease in inhibitory activity was noted with increasing time of addition of p53Δ30 (Figure 3A) until, at 120 min, there was virtually no effect, probably because replication is complete and only a single round of DNA synthesis takes place in these extracts (Blow and Laskey, 1986, 1988). Since addition of p53Δ30 at times up to 60 min also resulted in some decrease in overall levels of DNA synthesis achieved, these data suggest that p53Δ30 can arrest DNA synthesis even when replication forks are actively elongating. Although we cannot rule out the possibility that initiation is not synchronous and is therefore prevented by p53Δ30 in late replicating nuclei, this

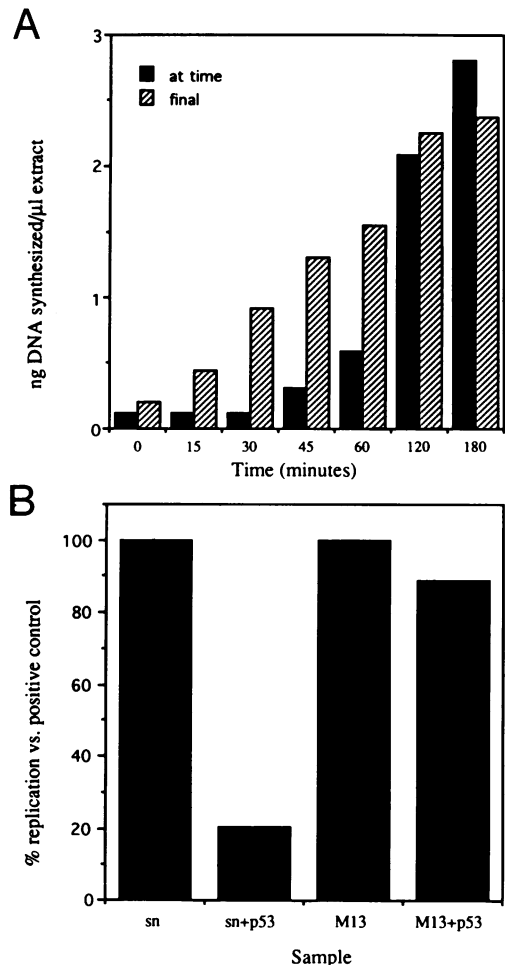


Fig. 3. p53Δ30 inhibits initiation of nuclear DNA replication. (A) Time course of p53Δ30 addition to *Xenopus* egg extract, showing that inhibition of sperm nuclear replication is maximal when p53Δ30 is added at or before the time of nuclear envelope assembly (Blow and Laskey, 1986; Blow and Watson, 1987). Hatched columns show the final level of DNA replication after a total 3 h incubation and filled columns show the amount of replication that had taken place before p53Δ30 was added to the extract at 'time'. (B) p53Δ30 reduces replication of *Xenopus* sperm nuclei (sn+p53) at least 5-fold from the levels observed without added p53Δ30 (sn), but does not prevent the formation of a second strand of DNA on a single-stranded M13 DNA template (compare M13+p53 with M13 alone).

explanation is less likely, since replication in this system is known to occur so rapidly that almost all replication forks must initiate synchronously (Blow and Watson, 1987; Mills *et al.*, 1989). However, these data show that the majority of inhibition occurs at or shortly after initiation.

To further define the activity of p53Δ30, the synthesis of DNA on a single-stranded M13 DNA template was compared with that of sperm chromatin in the presence and absence of p53Δ30 (Figure 3B). While replication of sperm nuclei was strongly inhibited, the second-strand synthesis reaction on M13 was not greatly affected by p53Δ30. This aphidicolin-sensitive reaction has been likened to lagging strand DNA synthesis (Mechali and Harland, 1982) and can occur in the absence of nuclear structure (Cox and Leno, 1990). Thus p53Δ30 does not interfere directly with polymerase $\alpha/\delta/\epsilon$ activity in this system nor can it act simply by coating DNA, but it

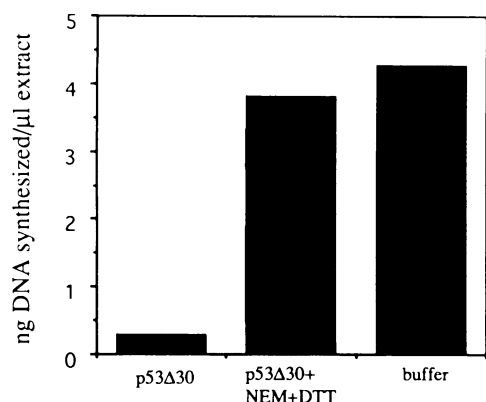


Fig. 4. Oxidation of p53Δ30 abolishes inhibitory activity. Oxidized p53Δ30 (p53Δ30+NEM+DTT) that has lost DNA binding activity (Hupp *et al.*, 1993) does not inhibit nuclear DNA replication when added to the *Xenopus* egg extract, whereas reduced p53Δ30 completely inhibits nuclear replication under the same conditions. The 'buffer' control contains the same volume of EBB as that added to the extract in the p53Δ30 sample.

does inhibit the synthesis of DNA at replication forks within nuclei.

Oxidation of p53Δ30 inactivates its inhibitory activity

Since p53Δ30 prevents nuclear DNA replication and full-length wtp53 does not, it was intriguing to determine whether the difference between these two forms of p53 lay in their different abilities to bind DNA. In the first instance, therefore, the DNA binding activity of p53Δ30 was ablated. Oxidation of p53 by agents such as *N*-ethylmaleimide (NEM) has been found to prevent p53Δ30 from binding to DNA (Hupp *et al.*, 1993). Therefore, we treated p53Δ30 with NEM, then quenched NEM activity using dithiothreitol (DTT) before addition to egg extract. Figure 4 shows that p53Δ30 oxidized by NEM/DTT treatment fails to inhibit replication, unlike untreated (reduced) p53Δ30. Addition of the same concentrations of NEM/DTT alone to the *Xenopus* egg extract had no significant effect on the level of DNA replication (data not shown). Incidentally, this result strongly suggests that it is p53Δ30 itself, rather than a non-specific co-purifying contaminant, that is exerting an effect on DNA synthesis. Therefore, the ability of p53Δ30 to block DNA replication *in vitro* is dependant on the protein existing in a reduced state and this correlates with its ability to bind DNA sequence-specifically. Hence, DNA binding by p53Δ30 is necessary for its ability to inhibit nuclear DNA replication.

Activation of wtp53 for DNA binding does not lead to inhibition of DNA replication

The p53Δ30 block to replication observed above may be due to the protein's enhanced double-strand DNA binding affinity compared with wtp53 (Hupp *et al.*, 1992). Full-length human p53 can be induced to bind DNA sequence-specifically with high affinity by incubating with an anti-C-terminal monoclonal antibody, PAb421 (Hupp *et al.*, 1992). Since DNA binding is necessary for inhibition of replication by p53, we addressed the question of whether such binding is sufficient to impose a block on DNA

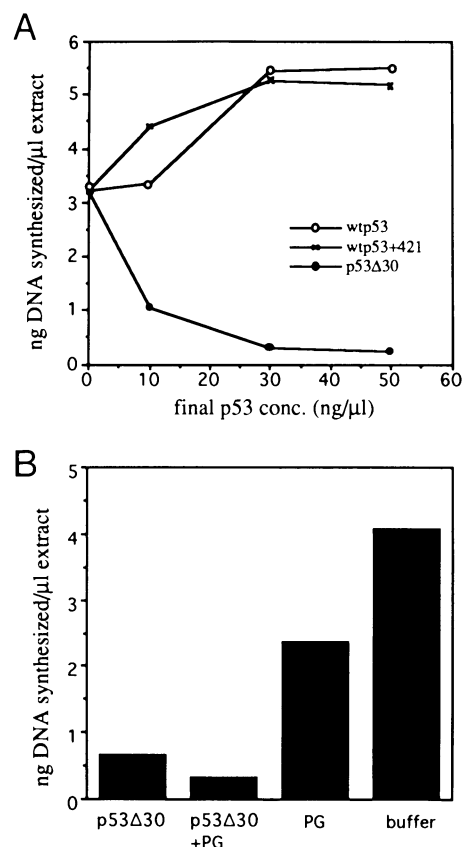


Fig. 5. Activation of wt p53 for DNA binding does not inhibit DNA replication. (A) wtp53 (open circles) is not activated for inhibition of nuclear replication by treatment with equimolar amounts of the anti-p53 (C-terminus) monoclonal antibody PAb421 (crosses), even though this treatment *in vitro* enhances its sequence-specific DNA binding capacity (Hupp *et al.*, 1992), whereas the constitutively activated form, p53Δ30, does block replication under identical conditions (closed circles). PAb421 alone added to replication mixes had no effect on the level of DNA synthesis (data not shown). (B) The p53 DNA binding consensus sequence polygrip (PG) does not competitively relieve inhibition of nuclear replication and may even lead to further inhibition when co-added with p53Δ30. Control samples included addition of PG DNA alone or EBB. All added volumes were the same so as to eliminate any dilution effects.

replication. We therefore compared replication of sperm nuclei in the presence of p53Δ30, wtp53 or wtp53 complexed with PAb421, to see if DNA binding was solely responsible for the inhibition of replication. Figure 5A clearly demonstrates that wtp53-421 behaved in the same way as wtp53 alone and did not inhibit replication of sperm nuclei *in vitro*, whereas p53Δ30 again showed concentration-dependent inhibition of replication. Hence, DNA binding by p53 is necessary, but not sufficient, for inhibition of nuclear DNA replication.

We further investigated whether DNA binding was the sole mechanism for the observed replication block, by competition analysis using an oligonucleotide containing the p53-specific DNA binding sequence PG (El-Deiry *et al.*, 1992). The data in Figure 5B show that co-incubation of p53Δ30 and PG DNA did not relieve the replication block. These results suggest that this block may require p53 to bind its target DNA sequence, but that additional mechanisms, such as protein-protein interactions between p53 (bound to DNA) and replication

proteins, might enhance the inhibitory effects of p53 on DNA replication.

Discussion

This paper aimed to distinguish between transcriptional and replication roles of p53 by examining the effects of purified human p53 on nuclear DNA replication in a cell-free replication system derived from activated *Xenopus* eggs (Blow and Laskey, 1986) that does not support transcription (Bachvarova and Davidson, 1966). This system is exciting in that it exploits a natural developmental phenomenon that allows experimental dissociation of these two fundamental processes without resorting to damaging, artefact-inducing drugs. It is also the only cell-free system to date known to support temporally and spatially regulated replication of eukaryotic nuclei (Blow and Laskey, 1986, 1988; Blow and Watson, 1987; Hutchison *et al.*, 1987; Mills *et al.*, 1989; Cox and Laskey, 1991), as opposed to viral DNA synthesis in soluble systems. The early *Xenopus* embryo undergoes 12 synchronous and very rapidly alternating S and M phase cell cycles without intervening G phases (Laskey, 1985), so it is likely that any endogenous damage check-point controls, such as that mediated by p53, remain latent until after the mid-blastula transition (MBT), when the cell cycle lengthens and incorporates G phases (Newport and Kirschner, 1982). *Xenopus laevis* is tetraploid and has two genes for p53 (Soussi *et al.*, 1987; Hoefer *et al.*, 1994). Although maternal stockpiles of p53 mRNA and protein are present in early development (Tchang *et al.*, 1993; Cox *et al.*, 1994; Hoefer *et al.*, 1994), it is probable that mechanisms exist in the rapidly dividing pre-MBT embryo to inactivate growth suppressor proteins such as p53, perhaps by dephosphorylation (Hupp *et al.*, 1993). By using the truncated form of human p53, which lacks the C-terminal casein kinase II phosphorylation site (Meek *et al.*, 1990), we have been able to overcome these putative inactivation mechanisms.

In this paper we observe that a C-terminal truncated form of p53, p53 Δ 30, which lacks part of the negative regulation domain and can bind DNA constitutively, inhibits nuclear DNA synthesis in a concentration-dependent manner in the *Xenopus* egg extract, whereas full-length wtp53 has no effect on nuclear DNA replication. The two forms of p53 were found to be equally stable during the course of these experiments, so the difference in replication effect was not due to differential degradation of wtp53 over p53 Δ 30. Both p53 Δ 30 and wtp53 inhibit synthesis of SV40 DNA in extracts of human HeLa cells. SV40 viral DNA replication in HeLa cell extract requires the activity of the essential viral replication protein, large T antigen. wtp53 is known to bind to T antigen and block its replication activities (Wang *et al.*, 1989; Friedman *et al.*, 1990) and we have shown that p53 Δ 30 equally interacts with T antigen (L.S.Cox, unpublished observations) to prevent viral DNA synthesis. The two experimental replication systems used in this paper therefore differ in that nuclear DNA replication in the *Xenopus* system uses normal eukaryotic synthetic machinery, while SV40 DNA synthesis depends on T antigen. This difference may account for the ability of wtp53 to inhibit SV40 but not nuclear DNA synthesis, as shown here. The concentration of p53 Δ 30 required to block

nuclear DNA replication is suggestive of a stoichiometric requirement for p53 in replication complexes; inhibition of SV40 replication by p53 binding to and inactivating T antigen requires very similar concentrations of p53. No detectable transcription takes place in the *Xenopus* egg extract, suggesting that the observed replication block does not require the transcriptional activity of p53. The absence of a transcriptional component to the replication block was verified by using the RNA polymerase inhibiting drug α -amanitin.

In human cells, full-length wtp53 is thought to exert the detected cell cycle block in response to DNA damage (Kasten *et al.*, 1991, 1992; Lu and Lane, 1993), so what is the relevance of inhibition by an artificially truncated form of the protein? An alternately spliced form of p53, which is nine amino acids shorter at the C-terminus, exists at 25–33% of the major p53 species in normal and transformed mouse cells (Han and Kulesz-Martin, 1992; Kulesz-Martin *et al.*, 1994), thus lacking the casein kinase II phosphorylation sites and possibly also lacking other allosteric regulation sites. This protein is preferentially present at times when DNA replication does not take place, i.e. during G₂ of the normal cell cycle and in G₁ when the DNA has been damaged by drug treatment (Kulesz-Martin *et al.*, 1994). It also appears probable that on DNA damage, wtp53 may change in conformation from a 'latent' form with weak affinity for DNA to an 'active' form that binds DNA more strongly (Hupp *et al.*, 1992; Lane, 1992), possibly by C-terminal modification, such as phosphorylation (Hupp *et al.*, 1993), alternative multimerization or even by controlled cleavage of the protein. By using p53 Δ 30, we have supplied an artificially activated form of p53 that cannot be inactivated by growth promoting factors in the egg extract. This form of p53 may reflect the activity of endogenous mammalian p53 in cells harbouring damaged DNA.

p53 Δ 30 was found to block nuclear DNA replication at an early stage, either at or soon after initiation, but it does not prevent synthesis of a second strand of DNA on a primed single-stranded M13 template. Therefore, p53 does not directly interfere with the DNA polymerases, and it appears from our data that replication forks structurally constrained within nuclei are the targets of p53 action. In favour of the hypothesis that p53 prevents initiation or early fork unwinding are the observations of Oberosler *et al.* (1993) and Brain and Jenkins (1994) that p53 can act to promote re-annealing of separated strands of DNA and RNA. Additionally, p53 binds to RP-A (Dutta *et al.*, 1993) and so may promote destabilization of the single-stranded regions at the replication fork and hence enhance re-association of the strands.

DNA binding appears critical for the ability of p53 to suppress growth (Friend, 1994), on the basis of structural (Cho *et al.*, 1994; Clore *et al.*, 1994) and mutational (Hollstein *et al.*, 1991) analyses of p53. Here we show that the ability of p53 Δ 30 to block DNA replication correlates with its DNA binding capacity, since oxidation leads to loss of both activities. This result may be taken to imply that the inhibition of DNA replication is exerted by p53 imposing a steric block to passage of replication forks. Therefore, it is possible that by binding to DNA, either in *cis* (on replicating DNA) or in *trans* (by association with its consensus sites in other regions of the

genome), p53 may be conformationally altered in such a way as to act more efficiently as an anti-helicase or to interact more strongly with proteins of the replication complex. Interestingly, wtp53, which is usually activated for DNA binding by incubation with the C-terminal antibody PAb 421, could not be activated to block DNA synthesis, suggestive of steric effects of the bound antibody that may prevent direct associations with replication proteins.

It is probable that p53 acts on the cell cycle in multiple ways to ensure that DNA replication is prevented on genotoxic insult. First, by modulating transcription of regulatory genes such as p21/WAF-1 (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Serrano *et al.*, 1993; Xiong *et al.*, 1993), the activity of cyclin-cdk kinase complexes is regulated and the cell cycle arrested either in G₁, at the G₁/S border or in S phase, via p21-mediated inhibition of cyclinE-cdk2, cyclinA-cdk2 or cyclin A-cdc2 respectively (Dulic *et al.*, 1994). If cells manage to progress into S phase in the presence of damage, then p21 induced by p53 can bind to and inactivate the essential replication protein PCNA (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994; Warbrick *et al.*, 1995). In addition to these transcriptional mechanisms, the data we present here strongly suggest that p53, when activated *in vivo*, can itself block DNA replication. By a combination of these three mechanisms, the cell should ensure that DNA replication cannot proceed until damage has been repaired or that the cell undergoes apoptosis rather than attempting to divide. In support of our results are the findings of Caelles *et al.* (1994), that show no requirement for either new RNA or protein synthesis for p53-dependent apoptosis.

In conclusion, in this paper we have managed to dissociate the putative roles of p53 in transcription and DNA replication by using a replication system in which measurable transcription does not take place and our results show that an additional mechanism for growth suppression by p53 may exist via a direct block on eukaryotic nuclear DNA replication.

Materials and methods

p53 expression and purification

Human p53 was expressed in *Escherichia coli* (Midgley *et al.*, 1992) or insect Sf9 cells infected with baculovirus containing cDNA for wtp53 or p53Δ30 (Hupp *et al.*, 1992). After biochemical purification on heparin-Sepharose and gel filtration, the proteins were microdialysed into EBB buffer (50 mM KCl, 50 mM HEPES-KOH, pH 7.4, 5 mM MgCl₂, 2 mM β-mercaptoethanol, 10% glycerol, 0.1% Triton X-100). There was no appreciable difference in the activity of p53 proteins from bacterial or baculovirus sources (data not shown).

Replication reactions

Xenopus egg extract was prepared essentially as described by Blow and Laskey (1986). Replication reactions were supplemented with an energy regenerating system (150 μg/ml creatine phosphokinase, 60 mM phosphocreatine), 100 μg/ml cycloheximide and 800 Ci/mmol [α -³²P]dATP (Amersham). Demembrated *Xenopus* sperm nuclei were prepared according to the method of Gurdon (1976). Single-stranded M13 DNA was prepared by standard methods (Sambrook *et al.*, 1989), RNase treated and purified by centrifugation on caesium chloride gradients. DNA templates were used at a final concentration of 5 ng/μl, and purified wtp53 or p53Δ30 was added to various final concentrations up to 40 ng/μl. The same volume of EBB was added to negative control samples (without p53) as the volume of p53 in EBB added to the experimental samples, to control for dilution and buffer effects. Where

appropriate, p53Δ30 was oxidized by treating with 5 mM NEM and then 5 mM DTT was added to quench the reaction. Reactions were carried out at 23°C for 3 h and incorporation of label determined by trichloroacetic acid precipitation followed by scintillation counting. A series of experiments using human p53 purified from baculovirus and *E. coli* expression systems showed inhibition of nuclear DNA replication at the same concentrations of p53 and representative examples are shown in the figures. Error bars are not shown, since *Xenopus* egg extracts vary from batch to batch in the absolute levels of DNA synthesis observed (see Table I in Blow and Laskey, 1986). SV40 DNA replication reactions were performed essentially as described in Wang *et al.* (1989), except that total reaction volumes were 10 μl. Antibody PAb421 against the C-terminus of p53 was purified on protein A-Sepharose and dialysed into EBB before mixing at equimolar concentrations with wtp53 or direct addition to the extract. PG DNA was prepared as previously described (Hupp *et al.*, 1992), but was not radioactively labelled. Where stated, the transcriptional inhibitor α-amanitin was used at a final concentration of 5 μg/ml.

Immunoblotting

Proteins were electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose and probed with undiluted tissue culture supernatant of the monoclonal antibody DO1 (Vojtesek *et al.*, 1992). Secondary horseradish peroxidase-conjugated rabbit anti-mouse antibody was used at 1:1000 dilution and visualized by the ECL technique.

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References

- Arai, N., Nomura, D., Yokota, K., Wolf, D.E.B., Shohat, O. and Rotter, V. (1986) *Mol. Cell. Biol.*, **6**, 3232–3239.
- Bachvarova, R. and Davidson, E.H. (1966) *J. Exp. Zool.*, **163**, 285–295.
- Blow, J.J. and Laskey, R.A. (1986) *Cell*, **47**, 577–587.
- Blow, J.J. and Laskey, R.A. (1988) *Nature*, **332**, 546–548.
- Blow, J.J. and Watson, J.V. (1987) *EMBO J.*, **6**, 1997–2002.
- Brain, R. and Jenkins, J. (1994) *Oncogene*, **9**, 1775–1780.
- Braithwaite, A.W., Sturzbecher, H.-W., Addison, C., Palmer, C., Rudge, K. and Jenkins, J.R. (1987) *Nature*, **329**, 458–460.
- Caelles, C., Helmborg, A. and Karin, M. (1994) *Nature*, **370**, 220–223.
- Cho, Y., Gorina, S., Jeffrey, P.D. and Pavletich, N.P. (1994) *Science*, **265**, 346–355.
- Clare, G.M., Omichinski, J.G., Sakaguchi, K., Zambano, N., Sakamoto, H., Appella, E. and Gronenborn, A.M. (1994) *Science*, **265**, 386–391.
- Cox, L.S. and Laskey, R.A. (1991) *Cell*, **66**, 271–275.
- Cox, L.S. and Leno, G.H. (1990) *J. Cell Sci.*, **97**, 177–184.
- Cox, L.S., Midgley, C.A. and Lane, D.P. (1994) *Oncogene*, **9**, 2951–2959.
- Crook, T., Marston, N.J., Sara, E.A. and Vousden, K.H. (1994) *Cell*, **79**, 817–827.
- Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T., Lees, E., Harper, J.W., Elledge, S.J. and Reed, S.I. (1994) *Cell*, **76**, 1013–1023.
- Dutta, A., Ruppert, J.M., Aster, J.C. and Winchester, E. (1993) *Nature*, **365**, 79–82.
- El-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W. and Vogelstein, B. (1992) *Nature Genet.*, **1**, 45–49.
- El-Deiry, W.S. *et al.* (1993) *Cell*, **75**, 817–825.
- Fields, S. and Yang, S.K. (1990) *Science*, **249**, 1046–1048.
- Friedman, P.N., Kern, S.E., Vogelstein, B. and Prives, C. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9275–9279.
- Flores-Rozas, H., Kelman, Z., Dean, F.B., Pan, Z.-Q., Harper, J.W., Elledge, S.J., O'Donnell, M. and Hurtwitz, J. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 8655–8659.
- Friend, S. (1994) *Science*, **265**, 334–335.
- Gannon, J.V. and Lane, D.P. (1987) *Nature*, **329**, 456–458.
- Gu, Y., Turck, C.W. and Morgan, D.O. (1993) *Nature*, **366**, 707–710.
- Gurdon, J.B. (1976) *J. Embryol. Exp. Morphol.*, **36**, 523–540.
- Halazonetis, T.D., Davis, L.J. and Kandil, A.N. (1993) *EMBO J.*, **12**, 1021–1028.

- Han, K.-A. and Kulesz-Martin, M.F. (1992) *Nucleic Acids Res.*, **20**, 1979–1981.
- Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993) *Cell*, **75**, 805–816.
- He, Z., Brinton, B.T., Greenblatt, J., Hassell, J.A. and Ingles, C.J. (1993) *Cell*, **73**, 1223–1232.
- Hoever, M., Clement, J.H., Wedlich, D., Montenarh, M. and Knochel, W. (1994) *Oncogene*, **9**, 109–120.
- Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. (1991) *Science*, **253**, 49–53.
- Hupp, T.R. and Lane, D.P. (1994) *Cold Spring Harbor Symp. Q. Biol.*, **LVIX**, in press.
- Hupp, T.R., Meek, D.W., Midgley, C.A. and Lane, D.P. (1992) *Cell*, **71**, 875–886.
- Hupp, T.R., Meek, D.W., Midgley, C.A. and Lane, D.P. (1993) *Nucleic Acids Res.*, **21**, 3167–3174.
- Hutchison, C.J. and Kill, I. (1989) *J. Cell Sci.*, **93**, 605–613.
- Hutchison, C., Drepaul, R.S., Gomperts, M. and Ford, C.C. (1987) *EMBO J.*, **6**, 2003–2010.
- Iwabuchi, K., Bartel, P.L., Li, B., Marraccino, R. and Fields, S. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 6098–6102.
- Kasten, M.B., Onkywere, O., Sidransky, D., Vogelstein, B. and Craig, R.W. (1991) *Cancer Res.*, **51**, 6304–6311.
- Kasten, M.B., Zhan, Q., El-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B. and Fornace, A.J., Jr (1992) *Cell*, **71**, 587–597.
- Kulesz-Martin, M.F., Lisafeld, B., Huang, H., Kisiel, N.D. and Lee, L. (1994) *Mol. Cell. Biol.*, **14**, 1698–1708.
- Lane, D.P. (1992) *Nature*, **358**, 15–16.
- Lane, D.P. and Crawford, L.V. (1979) *Nature*, **278**, 261–263.
- Laskey, R.A. (1985) *J. Embryol. Exp. Morphol.*, **89**, 285–295.
- Li, R. and Botchan, M.R. (1993) *Cell*, **73**, 1207–1221.
- Linzer, D.I.H. and Levine, A.J. (1979) *Cell*, **17**, 43–52.
- Lu, X. and Lane, D.P. (1993) *Cell*, **75**, 765–778.
- Maxwell, S.A. and Roth, J.A. (1993) *Oncogene*, **8**, 3421–3426.
- Mechali, M. and Harland, R.M. (1982) *Cell*, **30**, 93–101.
- Meek, D.W., Simon, S., Kikkawa, V. and Eckhart, W. (1990) *EMBO J.*, **9**, 3253–3260.
- Midgley, C.A., Fisher, C.J., Bartek, J., Vojtesek, B., Lane, D.P. and Barnes, D.M. (1992) *J. Cell Sci.*, **101**, 183–189.
- Mills, A.D., Blow, J.J., White, J.G., Amos, W.B., Wilcock, D. and Laskey, R.A. (1989) *J. Cell Sci.*, **94**, 471–477.
- Milner, J., Chan, Y.S., Medcalf, E.A., Wang, Y. and Eckhart, W. (1993) *Oncogene*, **8**, 2001–2008.
- Mulligan, L.M., Matleashewski, G.J., Scrabble, H.J. and Cavanee, W.K. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 5863–5867.
- Newport, J. and Kirschner, M. (1982) *Cell*, **30**, 675–686.
- Nigro, J.M. et al. (1989) *Nature*, **342**, 705–708.
- Obersler, P., Hloch, P., Ramsperger, U. and Stahl, H. (1993) *EMBO J.*, **12**, 2389–2396.
- Pietenpol, J.A. and Vogelstein, B. (1993) *Nature*, **365**, 17–18.
- Pietenpol, J.A., Tokino, T., Thiagalingam, S., El-Deiry, W., Kinzler, K. and Vogelstein, B. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 1998–2002.
- Reed, M., Wang, Y., Mayr, G., Anderson, M.E., Schwedes, J.F. and Tegtmeyer, P. (1993) *Gene Expression*, **3**, 95–107.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sang, B.-C., Chen, J.-Y., Minna, J. and Barbosa, M.S. (1994) *Oncogene*, **9**, 853–859.
- Serrano, M., Hannon, G. and Beach, D. (1993) *Nature*, **366**, 704–707.
- Soussi, T., Caron de Fromental, C., Mechali, M., May, P. and Kress, M. (1987) *Oncogene*, **1**, 71–78.
- Sturzbecher, H.-W., Brain, R., Maimets, T., Addison, C., Rudge, K. and Jenkins, J.R. (1988) *Oncogene*, **3**, 405–413.
- Subler, M.A., Martin, D.W. and Deb, S. (1994) *Oncogene*, **9**, 1351–1359.
- Takimoto, M., Sermsuvitayawong, K. and Matsubara, K. (1994) *Biochem. Biophys. Res. Commun.*, **202**, 490–496.
- Tchang, F., Gusse, M., Soussi, T. and Mechali, M. (1993) *Dev. Biol.*, **159**, 163–172.
- Vojtesek, B., Bartek, J., Midgley, C.A. and Lane, D.P. (1992) *J. Immunol. Methods*, **151**, 237–244.
- Waga, S., Hannon, G.J., Beach, D. and Stillman, B. (1994) *Nature*, **369**, 574–578.
- Wang, E.H., Friedman, P.N. and Prives, C. (1989) *Cell*, **57**, 379–392.
- Warbrick, E., Lane, D.P., Glover, D.M. and Cox, L.S. (1995) *Curr. Biol.*, **5**, 275–282.
- Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. (1993) *Nature*, **366**, 701–704.
- Zhang, W., Funk, W.D., Wright, W.E., Shay, J.W. and Deisseroth, A.B. (1993a) *Oncogene*, **8**, 2555–2559.
- Zhang, W., Shay, J.W. and Deisseroth, A.B. (1993b) *Cancer Res.*, **53**, 4772–4775.
- Zhang, W., Gou, X.-Y., Hu, G.-Y., Liu, W.-B., Shay, J.W. and Deisseroth, A.B. (1994) *EMBO J.*, **13**, 2535–2544.

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